RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ASSAY OF NALBUPHINE IN NALBUPHINE INJECTION

Dissertation Submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY

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In Partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

Reg. No: 261430212

Under the guidance of

Mr. V.SEKAR, M.Pharm.

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

J.K.K.NATTRAJA COLLEGE OF PHARMACY

KOMARAPALAYAM-638183

TAMILNADU APRIL-2016

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR ESTIMATION OF RELATED SUBSTANCE OF NALBUPHINE HCL IN NALBUPHINE HCL INJECTION"** submitted by the student bearing **Reg. No:261430212** to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS** was evaluated by us during the examination held on…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation "**RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR ESTIMATION OF RELATED SUBSTANCE OF NALBUPHINE HCL IN NALBUPHINE HCL INJECTION",** submitted to The Tamil Nadu Dr.M.G.R.Medical University, Chennai, was carried out by **Mr. ELAVARASU. K [Reg.No: 261430212],** for the Partial fulfillment of degree of **MASTER OF PHARMACY** in Department Of Pharmaceutical Analysis under direct supervision of Mr**.V.SEKAR, M.Pharm.,** Head **,** Department Of Pharmaceutical Analysis, J.K.K.Nataraja College of Pharmacy, Komarapalayam, during the academic year 2015-2016.

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This is to certify that the work embodied in this dissertation "**RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR ESTIMATION OF RELATED SUBSTANCE OF NALBUPHINE HCL IN NALBUPHINE HCL INJECTION" ,** submitted in partial fulfillment to The Tamil Nadu Dr.M.G.R. Medical University, Chennai, in the requirement for the award of degree of **MASTER OF PHARMACY** in Analysis, is a bonafide work carried out **Mr. ELAVARASU. K, [Reg. No. 261430212]** during the academic year 2015-2016, under my guidance and direct supervision in the department of Pharmaceutical analysis, J.K.K.Nataraja College of Pharmacy, Komarapalayam.

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DECLARATION

The work presented in this dissertation entitled **"RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR ESTIMATION OF RELATED SUBSTANCE OF NALBUPHINE HCL IN NALBUPHINE HCL INJECTION",** was carried out by me, under the direct supervision of **Mr. V.SEKAR, M.Pharm.,** Head, Department Of Pharmaceutical Analysis, J.K.K.Nataraja College of Pharmacy, Komarapalayam.

I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

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Mr. ELAVARASU. K

(2621430212)

Dedicated to

Almighty

My Beloved Parents,

&

My Family Members

CONTENTS

LIST OF ABBREVIATIONS USED

ICH	-	International Conference on Harmonization
USP	-	United States of Pharmacopoeia
λ	-	Lambda
µg/ml	-	Microgram per milliliter
ng /ml	-	Nanogram per milliliter
µl	-	Micro liter
ml	-	Milliliter
mM	-	Milli mole
nm	-	Nanometer
mm	-	Millimeter
%	-	Percentage
%RSD	-	Percentage of Relative Standard Deviation
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
pH	-	Negative Logarithm of Hydrogen Ion
Rt	-	Retention time
S.D	-	Standard Deviation
RP-HPLC	-	Reverse Phase –High Performance Liquid Chromatography
min	-	Minute
ml /min	-	Milliliter / minute
v / v	-	Volume /Volume
ml /min	-	Millilitre /Minute

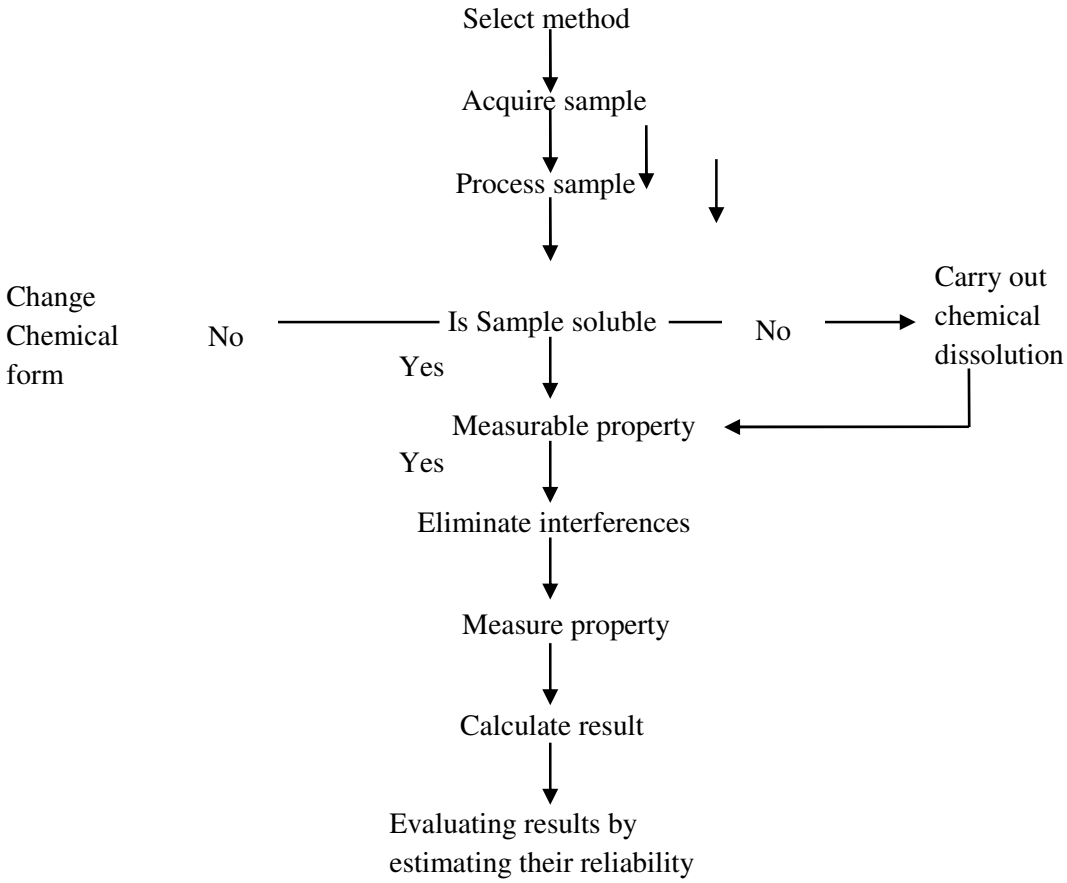
INTRODUCTION

Analytical chemistry is measurement science consisting of a set of powerful ideas and methods that are useful in all fields of science and method. it is used to study the chemical composition, structure and behavior of compounds. The purpose of chemical analysis is together and interpret chemical information that will be the value of society in a wide range of contexts. involves the application of a range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information on the nature of components.[1,2]

Qualitative analysis is the identification of elements and compounds present in a sample. Quantitative analysis is the determination of the absolute or relative amounts of elements or compounds present in a sample.

Structural analysis is the determination of the special arrangement of atoms in an element or molecule or the identification of characteristic groups or atoms.

A typical quantitative analysis[2] involves the sequence of steps shown in the following flow diagram (figure-1)



High Performance Liquid Chromatography [3,4,5,6,7]

High performance liquid chromatography is the fastest growing analytical technique for the analysis of the drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for an analysis of many drugs in both dosage forms and biological fluids. HPLC was developed in the late 1960s and 1970s. Today it is widely accepted separation technique for both sample analysis and purification in variety of areas.

Guide to Liquid Chromatography Mode Selection

Selection of chromatography mode is based upon the analyte polarity, solubility and ionic nature. [7] A guide to liquid chromatography mode selection is represented in figure-2

Figure - 2 Guide to Liquid Chromatography Mode Selection Reverse Phase HPLC

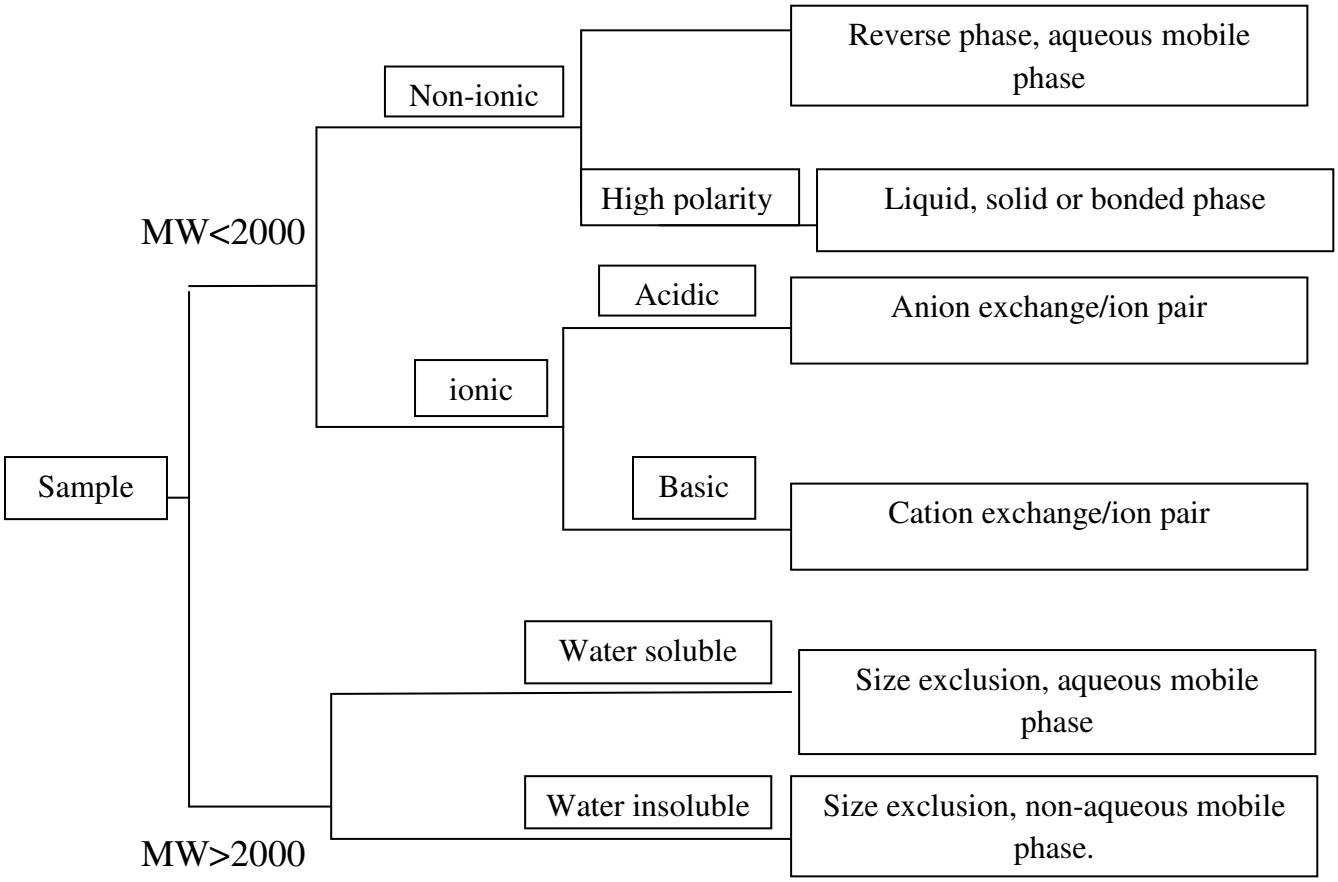
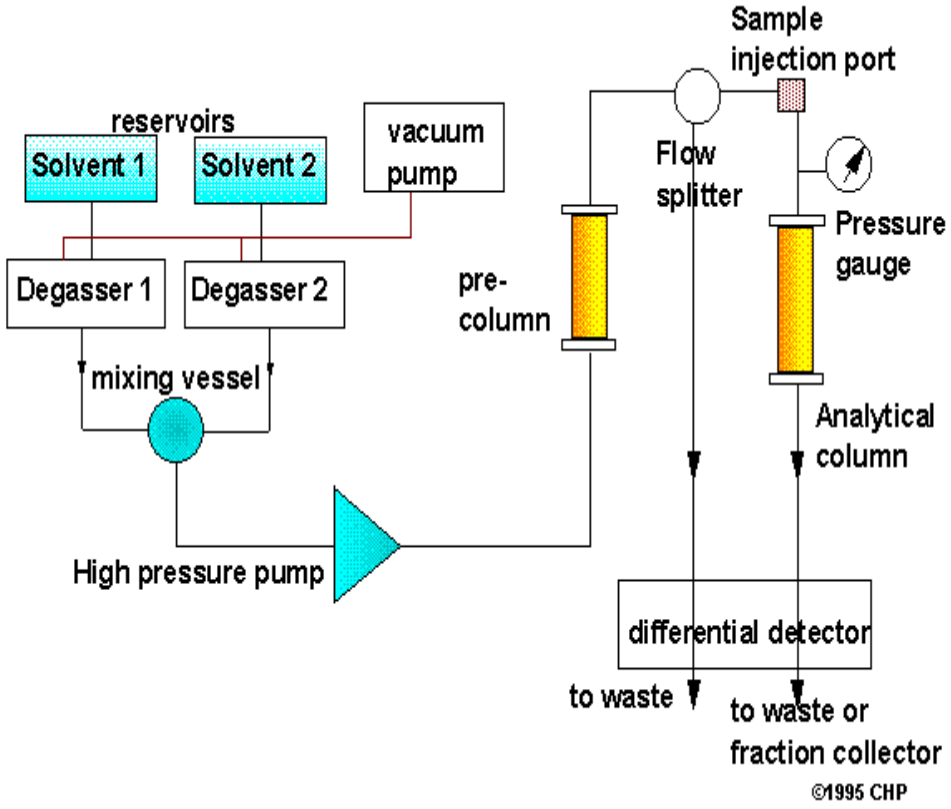


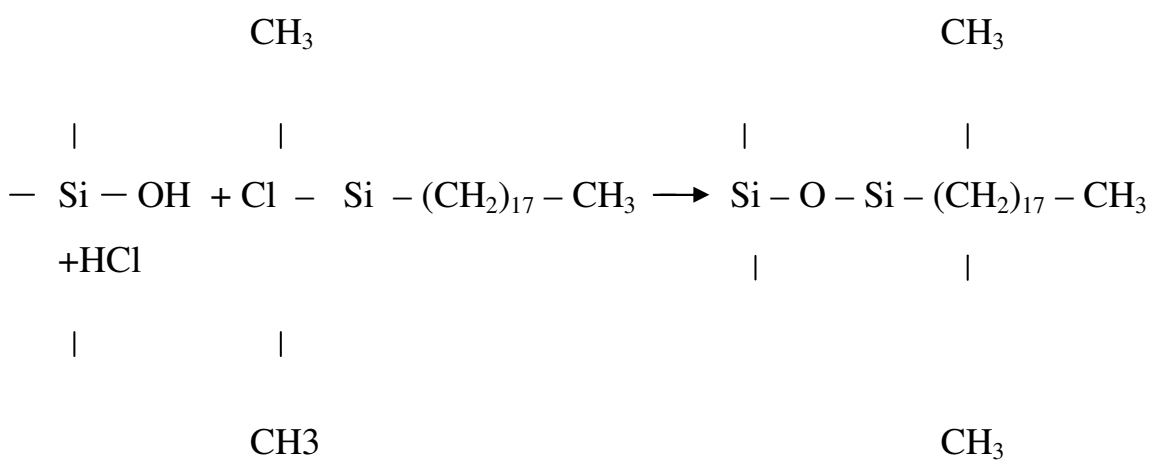
Figure - 3 Block diagram showing the components of an HPLC instrument



Reverse phase column packing

The liquid-liquid partition chromatography uses a stationary phase consisting of liquid layer adsorbed to a surface of silica or alumina. In case of bonded phase or reverse phase HPLC uses a stationary phase consisting of an organic moiety chemically bonded to the surface of silica through the surface silanol groups. Since the organic moieties are generally long-chain hydrocarbons, the mobile phases are generally polar. In this mode, the more polar solutes are eluted first while the non-polar compounds are eluted later. The silanol group can react with a chlorosilane group to form the popular (ODS & C_{18}) octadecylsilane packing.

E.g. Octadecylsilane (ODS or C_{18}).



For silica based bonded materials, the suitable working pH range is 2 - 8. At pH values of less than 2, the Si-C bond is attached and at the higher pH values, hydrolysis of the siloxane takes place which leads to degradation or destruction of the packing. In most of the applications of RP-HPLC, elution is carried out with highly polar solvents such as methanol, acetonitrile or tetrahydrofuran in various concentrations. [8]

Reverse phase mobile phase

The primary constituent of RP-HPLC mobile phase is water. Water miscible solvents such as methanol, ethanol, acetonitrile, dioxane, tetrahydrofuran and dimethyl formamide are added to adjust the polarity of the mobile phase. Additionally acids, bases, buffers and/or ionic surfactants are added. The water should be of high quality, either distilled or demineralised water.

The most widely used organic modifiers are methanol, acetonitrile and tetrahydrofuran. Methanol and acetonitrile have comparable polarities but acetonitrile is an aprotic solvent. Ethanol, 1-propanol and 2-propanol are also useful but less polar than methanol. Dioxane, tetrahydrofuran are aprotic solvents that are less polar than acetonitrile. Reverse phase mobile phases are generally non-flammable due to high water content. Degassing is quite important with reverse phase mobile phases.[8]

ANALYTICALMETHOD DEVELOPMENT [1,9]

Establishing an accurate assay procedure for each ingredient of complex dosage formulation containing several therapeutically and chemically compatible drugs with very similar chemical nature is a critical process. The presence of excipients, additives and decomposition products further complicates the analysis. Therefore analytical development is done for few drugs where no compendial method is available.

Method development is done for:

- New drug products
- Already existing products

Methods are developed for new products when no official methods are available and for already existing products to reduce the cost and time for better precision and ruggedness.

STEPS OF METHOD DEVELOPMENT[1]

It starts with the documentation of the developed studies. All the data related to these studies are established and recorded in laboratory notebook.

1. ANALYTICAL STANDARD CHARACTERIZATION

All the known information about the drug or analyte and its structure is collected such as its physical and chemical properties, toxicity, purity, hygroscopic nature solubility and stability.

a. The standard analyte is obtained. Necessary arrangement is made for proper storage in refrigerator, desiccators and freezer.
b. When multiple components are to be analyzed in the sample matrix the number of components are noted, data is assembled and the availability of standards for each one is determined.
c. Special attention to be taken when sample is in less quality.
d. Only the methods which are compatible with sample stability are considered.

2. METHOD REQUIREMENTS

The objectives of method are defined. The required detection limits, linearity, range, accuracy and precision are defined.

3. LITERATURE SEARCH AND RESEARCH METHODOLOGY

Carry out the literature survey for all types of information to the analyte. Literature is done for synthesis, physico-chemical properties, solubility and relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency compendia such as USP/NF, AOAC publications are reviewed along with chemical abstract service (CAS) automated computerized literature searches.

a. If any reported methods from the literature are adaptable to the current laboratory setting and future needs are determined.
b. Using information in the literature and prints, methodology is adapted. The methods are modified wherever necessary; acquire additional existing methods for in house analytes and samples.
c. If there are no prior methods for the analytes in the literature, the compounds that are similar and chemical properties are investigated and are worked out.

4. INSTRUMENTAL SET UP AND INITIAL STUDIES

a. The required instrument is set up. Installation, operational and performance of instrumentation using laboratory standard operating procedure are reviewed.
b. Always new consumables (solvents, filter and gases) are used.
c. The analyte standard in a suitable injection/introduction solution and in known concentration and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (example: bulk drug) then it is possible to start work with the actual sample.
d. Feasibility of method with regards to the analytical figures of merit obtained is evaluated.

5. OPTIMIZATION

During optimization one parameter is changed at a time and set of conditions are isolated rather than using a trial and error approach. Work has been done from an organized methodological plan and every step is documented in case of dead ends.

6. DOCUMENTATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLE

The sample solution should lead to absolute identification of the peak of interest apart from all other matrix components.

7. EVALUATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLE

The sample solution should lead to absolute identification of the peak of interest apart from all other matrix components.

8. DETERMINATION OF PERCENT RECOVERY OF ACTUAL SAMPLE AND DEMONSTRATION OF QUANTITATIVE SAMPLE ANALYSIS.

Percentage recovery was determined by adding authentic standard analyte into a sample matrix. Reproducibility of recovery from sample to sample and has been determined. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with high degree of certainty.

9. ANALYTICAL METHOD VALIDATION [10, 11, 12, 13, 35, 36]

Validation is a key process for effective Quality Assurance "Validation is establishing documented evidence which provides a high degree of assurance that a specific process or equipment will consistently produce a product or result meeting its predetermined specifications and quality attributes.

The validation guidelines recommended from ICH (International Conference on Harmonization) consists characteristics for consideration during the validation of analytical procedures included as part of registration applications within EC, Japan and USA.

9.1 Type of analytical procedures to be validated

Validation of analytical procedures is directed to the four most common types of analytical procedures.

1. Identification test.
2. Quantitative test for impurities content.
3. Limit test for the control of impurities
4. Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

Assay procedures are intended to measure the analyte present in given sample, assay represent a quantitative measurement of the major component(s) in the drug sample.

9.2 Objective of validation [14, 15]

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality, purity, safety and efficacy. Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

9.3 Benefits of Validation:

a. Produces quality products.
b. Helps in process improvement technology transfer, related product validation, failure investigation, and increased employee awareness.
c. Cost reduction by increasing efficacy, few reject, longer equipment life, production of cost effective products.
d. Helps in optimization of process or method.
e. Regulatory affairs department approved the products for export.

10.0 VALIDATION AS DEFINED BY DIFFERENT AGENCIES

1. USFDA:

According to this "Validation is the process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

2. WHO:

Defines Validation as an action of providing any procedure, process, equipment, material, activity or system actually leads to the expected results.

3. EUROPEAN COMMITTEE:

Defines Validation as an action of providing in accordance with the principles of GMP that any procedure, process, material, activity or system actually lead to expected results. This process consists of establishment of the performance characteristics and the limitations of the method.

11.0 Method performance parameters are determined using equipment that is:

1. Within specification.
2. Working correctly.
3. Adequately calibrated.

12.0 Method validation is required when:

1. A new method is developed.
2. Revision of established method.
3. When established methods are used in different laboratories and by different analysts.
4. Comparison of methods.
5. When quality control indicates method changes.

13.0 Typical analytical parameters used in assay validation include:

13.1 Precision
13.2 Accuracy
13.3 Linearity
13.4 Range
13.5 Ruggedness
13.6 Robustness
13.7 Limit of detection
13.8 Limit of quantitation
13.9 Selectivity
13.10 Specificity

13.1 PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed at the variance, standard deviation or coefficient of variation of a series of measurements.

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Precisionisdeterminedbyusingthemethodtoassayasampleforasufficientnumberof times to obtain statistically valid results (i.ebetween6-10).

$$\%RSD = \frac{STDdevx100\%}{Mean}$$

13.2 REPEATABILITY

It express the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra - assay precision. It should be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g. three concentration/three replicates each) or a minimum of determinations at 100% of the test concentration.

13.3 INTERMEDIATE PRECISION

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc.

13.4 REPRODUCIBILITY

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance inclusion of procedures in Pharmacopoeias.

13.5 ACCURACY

"Accuracy is a measure of the closeness of test results obtained by a method to the true value."

Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.

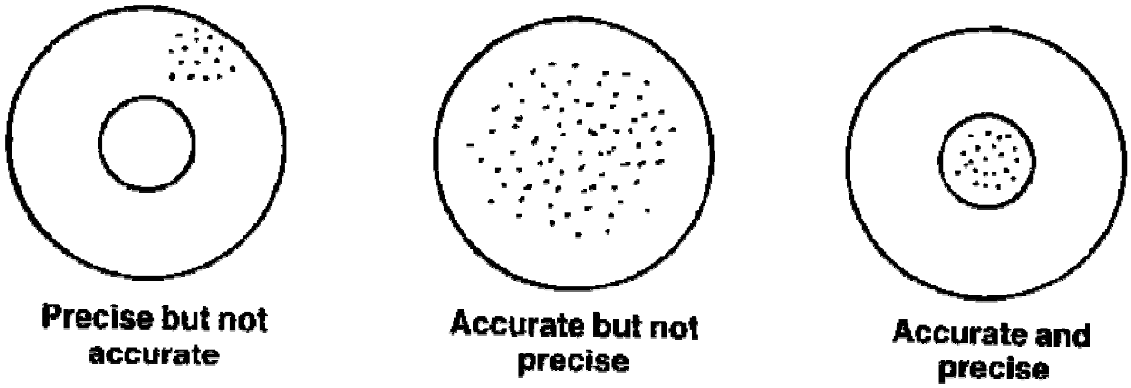


Figure-4

Errors in measurement can be divided into two general categories: systematic errors and random errors.

Systematic errors result from sources that can be traced to the methodology, the instrument or the operator and affect both the accuracy and the precision of the measurement.

Random errors only affect the precision and are difficult to eliminate, because they are the result of random fluctuations in the measured signal, due to noise and other factors.

Whilst systematic errors are proportional to the sum of individual contributions, random errors are proportional to the root of the sum of the squares of the individual contributions. Thus, the imprecision of the entire procedure is often dominated by the random errors of the most imprecise step.

13.6 LINEARITY

This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range. Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e. least mean squares) vs. analyte concentration.

13.7 RANGE

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (i.e. where more than one range is involved, as shown below) and is normally expressed in the same units as the test results.

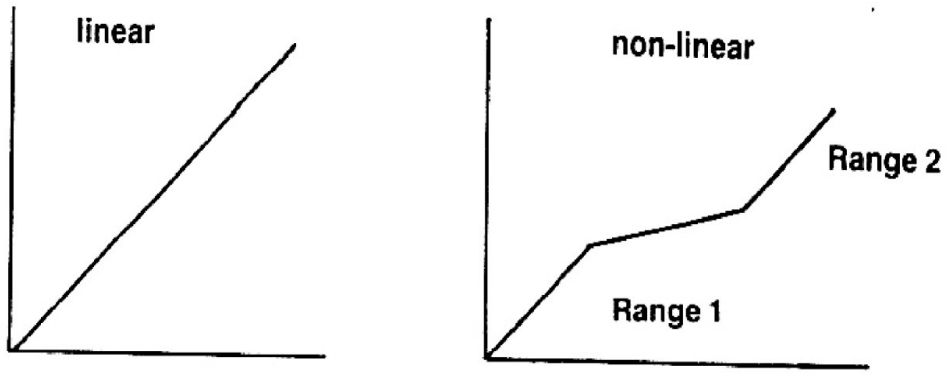


Figure- 5

13.8 RUGGEDNESS

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst.)

13.9 ROBUSTNESS

Robustness is depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. It is the measure of capacity of an assay to remain by small but deliberate variations in method parameters and provide an indication of its reliability in normal usage degradation and variation in chromatography column, mobile phases and inadequate method development are common causes of lack of robustness.

Examples of typical variations are

- Stability of analytical solutions
- Extraction time
- Influence of variation of pH in a mobile phase
- Influence of variation in mobile phase composition
- Different columns
- Temperatures
- Flow rate

13.10 LIMIT OF DETECTION

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos.

The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 2:1 and is confirmed by analyzing a number of samples near this value (6) using the following equation. The signal-to-noise ratio (5) is determined by:

$s = H/h$

Where H = height of the peak corresponding to the component

h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Since the limit of detection is dependent on the signal-to-noise ratio, it can be improved by enhancing the analyte signal and reducing the detector noise. The signal (i.e. peak height) can be increased by selecting the optimum monitoring wavelength, increasing the injection volume or mass (below signal or column

saturation), increasing the peak sharpness with high efficiency columns and by optimizing the mobile phase. For absorbance detectors, longer path lengths in the flow cell enhance sensitivity though often to the detriment of post column dispersion.

Noise can be reduced by using high sensitivity detectors with low noise and drift characteristics, slower detector response time, mobile phases with low absorbance and pumps with low pulsation.

13.11 LIMIT OF QUANTITATION

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

It is quoted as the concentration yielding a signal-to-noise ratio of 1 0: 1 and is confirmed by analyzing a number of samples near this value.

13.12 SELECTIVITY AND SPECIFICITY

Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

Specificity for an assay ensures that the signal measured comes from the substance of interest and that there is no interference from excipients and/or degradation products and/or impurities.

Determination of this can be carried out by assessing the peak identity and purity.

Diode array detectors can facilitate the development and validation of HPLC assays. Spectral data obtained from diode array detectors, effectively supplement the retention time data for peak identification, also spectral manipulation often provides information about the peak purity. The table below lists several of the techniques available for assessing peak identity and purity.

The purity index is a measure of the peak's relative purity, measured using a full comparison of spectral data for the leading and training edge of the peak Figure 4. A value of 1.5 is commonly accepted to indicate a pure peak but >1.5 would indicate the presence of an impurity, (9) as shown in Figure 6 & 7.

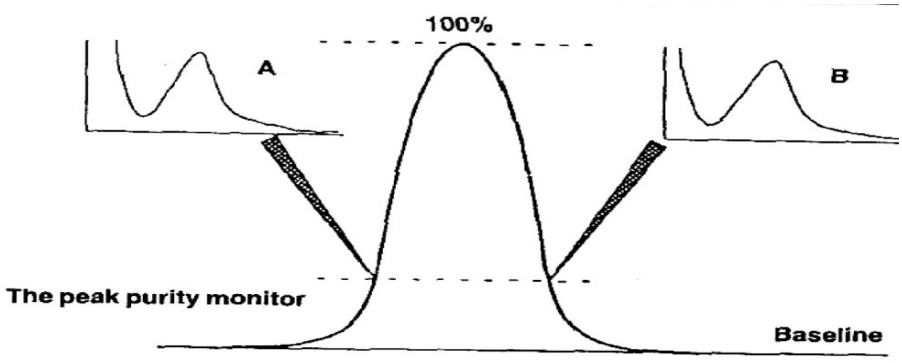
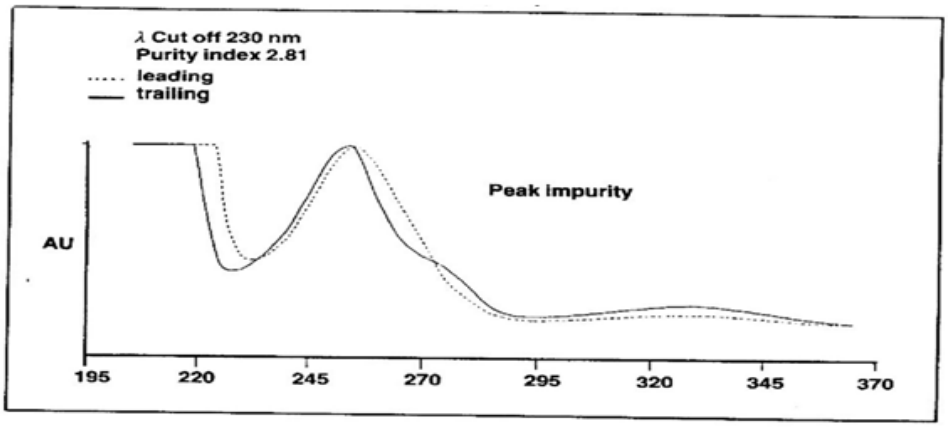


Figure- 6

Spectral comparison showing peak purity



Comparison of reference versus sample apex spectra for peak identity

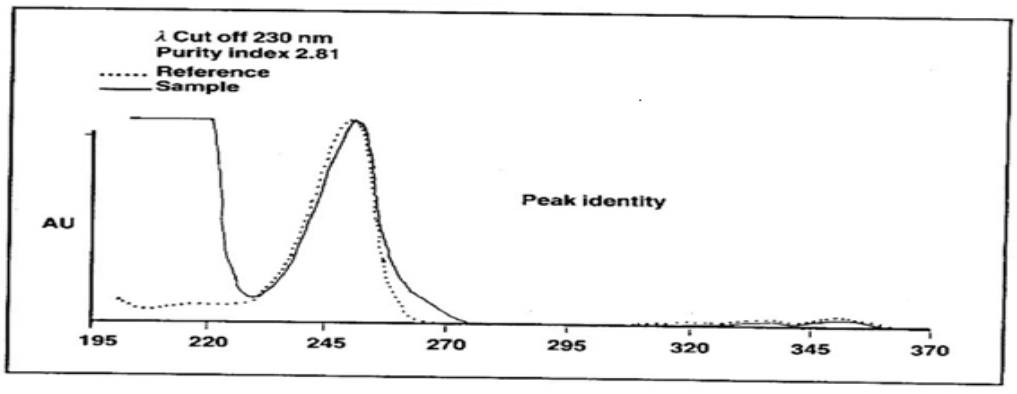


Figure-7

14.0 SYSTEM SUITABILITY TESTS (SST)

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to perform within the validated limits.

The simplest form of an HPLC system suitability test involves a comparison of the chromatogram trace with a standard trace. This allows a comparison of the peak shape, peak width, and baseline resolution.

Alternatively these parameters can be calculated experimentally to provide a quantitative system suitability test report:

14.1 Number of theoretical plates (efficiency)

14.2 Capacity factor

14.3 Separation (relative retention)

14.4 Resolution

14.5 Tailing factor

14.6 Relative Standard Deviation (Precision)

These are measured on a peak or peaks of known retention time and peak width.

15.0 PLATE NUMBER OR NUMBER OF THEORETICAL PLATES (N)

This measures the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP at half the Height.

An equation used to calculate 'N' is

$$\mathbf{N = 5.54[t/w_{h/2}]^2}$$

Where

$W_{h/2}$ = peak width at 1/2 peak height Wb = peak width at base

t = retention time of peak

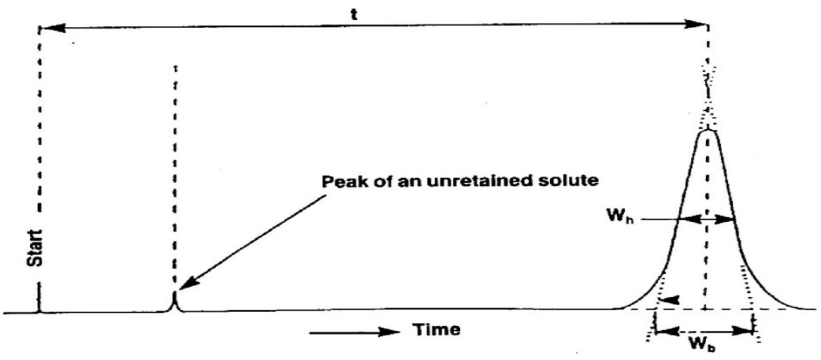


Figure- 8

Therefore the higher the plate number the more efficient the column.

The plate number depends on column length - i.e. the longer the column the larger the plate number. Therefore the column's efficiency can also be quoted as:

Either- as the plate height (h), or the height equivalent to one theoretical plate (HETP).

h= L/n

Where L = length of column n

Or - as plates/meter.

16.0 CAPACITY FACTOR (CAPACITY RATIO) K

This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).

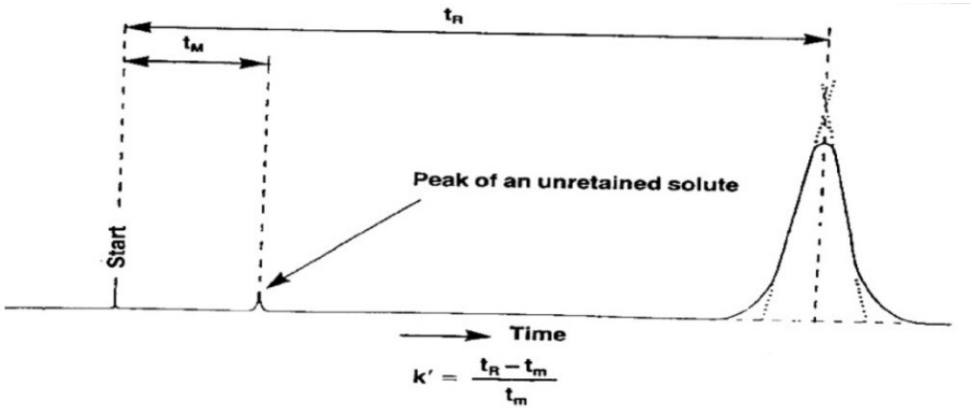


Figure- 9

k' is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (i.e. flow rate) and therefore ensures greater reproducibility from run to run. In practice the k value for the first peak of interest should be >l to assure that it is separated from the solvent.

17.0 SEPARATION FACTOR (RELATIVE RETENTION)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

Therefore considering peaks A and B

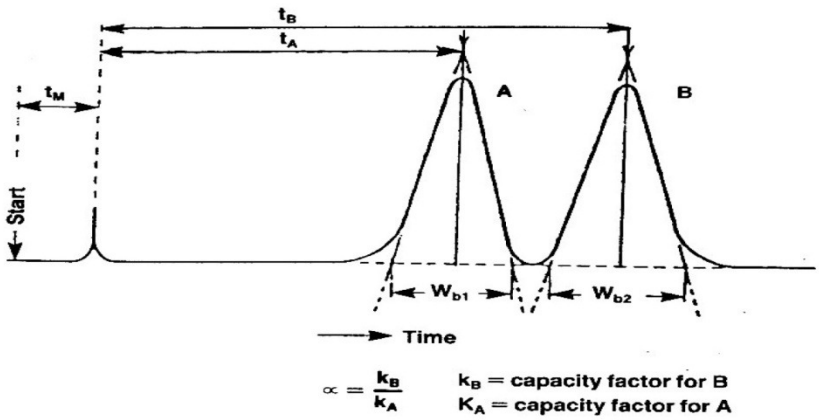


Figure- 10

k for the later peak is always placed in the numerator to assure a value >l. If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition and specified temperature, regardless of the instrument used.

18.0 PEAK RESOLUTION R

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb).

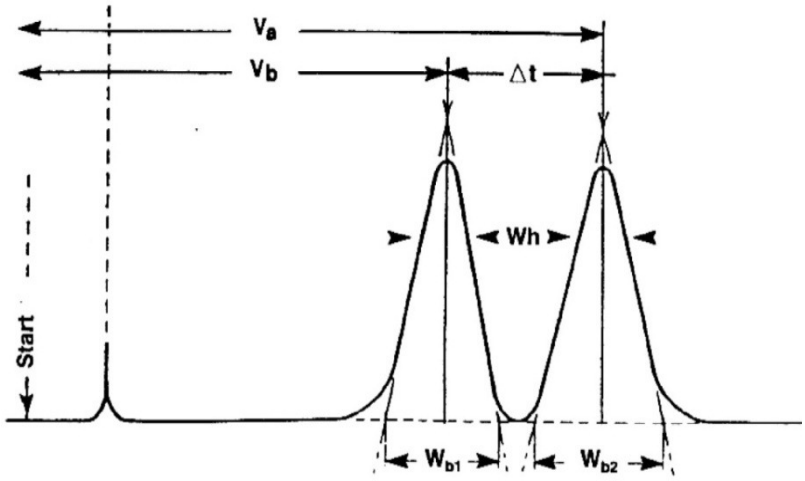


Figure- 11

19.0 TAILING FACTOR T

This is a measure for the asymmetry of the peak.

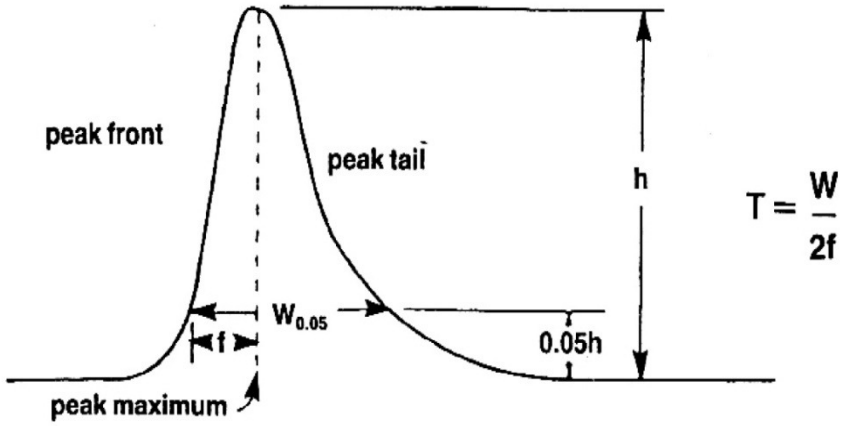


Figure- 12

20.0 RELATIVE STANDARD DEVIATION OR PRECISION

For an HPLC system this would involve the reproducibility of a number of replicate injections (i.e. 6) of an analytical solution.

The USP requires that unless otherwise specified by a method:

if a relative standard deviation of <2% is required then five replicate injections should be used. if a relative standard deviation of >2% is required then six replicate injections should be used Factors which could affect the precision of an HPLC system are shown in table 1.

Table No. 1

Precision	Controlling Factors
Retention time	Pump flow and composition precision Column temperature Mobile phase composition
Peak area	Auto sampler: inj mode, inj volume Pump: flow, pulsation Detector: noise and drift, response Data system: sampling rate, integration parameters

In most cases the system's Relative Standard Deviation is required; deciding which of the other tests are required is not straightforward. To assist with the decision it has been suggested that those parameters which have an effect on the system precision should be used. For instance the resolution of two peaks with similar retention times should be quoted, because, if it is below a critical value, the precision will be affected.

In addition "diode array" detectors allow for the determination of the relative purity factor typically called: Peak Purity.

The retention time precision is important, because not only is retention time the primary method for peak identification, but also variations can indicate problems within the LC system (i.e. with the piston seals, check valves etc). Use of a column oven can overcome laboratory temperature variations, which is the most common cause of retention time drift.

The most dominant factor controlling the repeatability of peak area is the auto sampler's precision, though the effect of noise and integration parameters will become more significant with small peaks

21.0 METHODS USED FOR THE EXAMINATION OF PHARMACEUTICAL MATERIAL MAY BE BROADLY CLASSIFIED AS:

CLASS A: Tests designed to establish identity, whether of bulk drug substances or of a particular ingredient in a finished dosage form.

CLASS B: Methods designed to detect and quantitate impurities in a bulk drug substance or finished dosage form.

CLASS C: Methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form,

CLASS D: Methods used to assess the characteristics of finished dosage forms such as dissolution profile and content uniformity.

LITERATURE REVIEW

Systematic literature survey is the main basis for the planning of any scientific work and due to the same reasons here the review of literature regarding estimation of *Nalbuphine HCl* in Tablet dosage formulation.

1. Khalid A. Attia ***et al.,*** have reported "A simple, sensitive, stability-indicating HPLC method was developed and validated for the quantitative determination of nalbuphine hydrochloride in presence of its degradation product.The analysis was carried out on a BDS Hypersil C18 (250 X 4.6 X 5µm particle size) using a mobile phase consisting of 5 mM sodium acetate buffer; pH 5.5: acetonitrile (40:60, v/v). The analysis was performed at ambient temperature with a flow rate of 1 ml/min and UV detection at 210 nm. The method showed good linearity over the concentration range of 1-15 µg/ml with a lower detection limit of 0.243 and quantification limit of 0.737 µg/ml. The proposed method can selectively analyse the drug in presence of up to 87% of its oxidative degradate with mean recovery± RSD% of 100.08±0.678.The method was validated and successfully applied for determination of nalbuphine in its commercial preparation and the obtained results were statistically compared with those of the reported method by applying t-test and F-test at 95% confidence level and no significant difference was observed regarding accuracy and precision..

2. Mary Ann Quarry et.al., A stability-indicating liquid chromatographic method has been developed for the determination of nalbuphine hydrochloride,
methylparaben, and propylparaben in nalbuphine hydrochloride injection. Reversed-phase chromatography was carried out using a mobile phase containing 0.05 % trifuoroacetic acid, acetonitrile, and tetrahydrofuran. Quantitation was achieved with UV detection at 280 nm. Validation data for linearity, accuracy, precision, specificity, and robustness are presented. The chromatographic

system resolves nalbuphine from synthetic impurities and degradation products.

3. Mary Ann Quarry ***et al***A method for the **Determination** of impurities and degradation products in nalbuphine hydrochloride injection by gradient elution high performance liquid chromatography (HPLC) is reported. Reversed phase gradient elution chromatography was carried out using a mobile phase containing 0.05% trifluoroacetic acid, acetonitrile, and tetrahydrofuran. Validation data for linearity, accuracy, precision, robustness, detection limit, and quantitation limit are presented. The chromatographic system resolves nalbuphine from related substances with corrections made for differences in detector response at the specified wavelength.

4. Louise M. Dubé, Nicole Beaudoin ***et al.,*** A rapid, selective and reproducible high-performance liquid chromatographic assay with electrochemical detection was developed for the determination of nalbuphine in human plasma. The method involves extraction with chloroform—isopropanol at pH 9.4, back-extraction into dilute phosphoric acid and reversed-phase chromatography on a μBondapak phenyl column. The recovery of nalbuphine and naltrexone (internal standard) was greater than 90%. Calibration curves were linear over a concentration range of 3–36 ng/ml with coefficients of variation, within-day or between-day, not exceeding 8% at any level. Although the limit of detection was 0.3 ng/ml based on a signal-to-noise ratio of 3, the reliable limit of quantitation was 1 ng/ml (coefficient of variation 12%) using 1 ml of plasma. The dual-electrode detector was operated in the screening mode of oxidation (electrode 1, 0.3 V and electrode 2, 0.6 V), providing a greater specificity and reducing background noise. This procedure was applied to a large number of clinical samples in an intravenous dose-range pharmacokinetic study in patients.and dispersion.

5. Mary Ann Quarrya, Reed C. Williamsa & Dolores S. Sebastiana A method for the determination of impurities and degradation products in nalbuphine hydrochloride injection by gradient elution high performance liquid

chromatography (HPLC) is reported. Reversed phase gradient elution chromatography was carried out using a mobile phase containing 0.05% trifluoroacetic acid, acetonitrile, and tetrahydrofuran. Validation data for linearity, accuracy, precision, robustness, detection limit, and quantitation limit are presented. The chromatographic system resolves nalbuphine from related substances with corrections made for differences in detector response at the specified wavelength.

6. **M. M. Fouad et.al**., Three simple, sensitive and reproducible spectrometric methods for the selective determination of nalbuphine–HCl in presence of its oxidative degradate were investigated. The first method depended on the quantitative densitometric evaluation of thin layer chromatograms of the drug at 284 nm using chloroform–methanol–acetic acid (7:3:0.05 v/v/v) as a mobile phase, in a concentration range of 10-30 µg/spot. The second one used the pH induced difference absorbance (ΔA) between 0.1M NaOH and 0.1 M HCl drug solutions at 299 nm to determine 20-160 µg mL-1 of the drug. The third method was a bivariate calibration algorithm for the determination of nalbuphine–HCl over concentration range of 20-200 µg mL-1 . The proposed methods selectively analysed the drug in presence of up to 80% of its oxidative degradate with mean recoveries of 100.63±1.03 for densitometric method and up to 90% with recoveries of 99.97±1.16 and 100.09±1.47% regarding the two other methods, respectively. The three proposed methods were successfully applied to analyse nalbuphine– HCl in its preparations, the results obtained were statistically analysed and found to be in accordance with those given by the compendial method.

AIM AND OBJECTIVE OF WORK

The drug analysis plays an important role in the development of drugs, their manufacture and the therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets the required specification. The number of drugs and drug formulations introduced in to the market has been increasing at an alarming rate. These drugs or formulation may be either in the new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms.

The multi component dosage form proves to be effective due to the combined mode of action on the body. The complexity of dosage forms including the presence of multiple drug entities posses considerable challenge to the analytical chemist during the development of assay procedure. The estimation of individual drugs in these multi component dosage forms becomes difficult due to cumbersome extraction or isolation procedures.

For the present study of *Nalbuphine HCl* was selected. The extensive literature survey carried out and revealed that there is only few method reported for the estimation of this drug. Hence an attempt was made to develop a specific, precise, accurate, linear, simple, rapid, validated and cost effective RP-HPLC method for the estimation of this drug in Pharmaceutical dosage forms.

PLAN OF WORK

To develop and validate an effective RP – HPLC method for the estimation of *Nalbuphine HCl* in bulk and its pharmaceutical dosage forms.

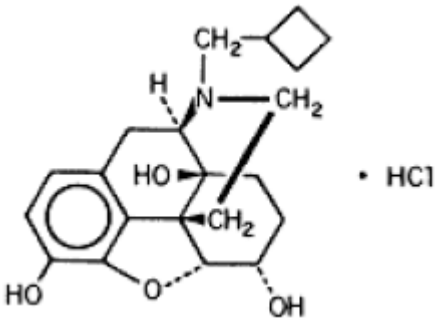
So ,the plan of work for the designed study was as follows:

- Gathering physical chemical properties of drug
- From the UV- analysis ,selection of λ max
- Selection of chromatographic condition
  - Selection of stationary phase
  - Selection of mobile phase
  - Selection of flow rate
  - Selection of Initial separation condition
- Optimization of chromatographic condition
- Validation of proposed method
- Applying developed method to the marketed formulation.
- Summarize methodology, finalize documentation.

DRUG PROFILE

Nalbuphine HCl

Structure :



Chemical name **:** 17-(cyclobutylmethyl)- 4,5α-epoxymorphinan-3, 6α, 14-triol hydrochloride

Description **:** **White to slightly off-white powder**

Molecular formula **:** $C_{21}H_{27}NO_4 \cdot HCl$

Molecular mass **:** 393.91 g/mol

Bioavailability **:** 81 %

Half- life **:** 5 hours

Category **:** semi-synthetic opioid agonist-antagonist analgesic of the phenanthrene series

M.O.A **:** The exact mechanism of action is unknown, but is believed to interact with an opiate receptor site in the CNS (probably in or associated with the limbic system). The opiate antagonistic effect may result from competitive inhibition at the opiate receptor, but may also be a result of other mechanisms. Nalbuphine is thought primarily to be a kappa agonist. It is also a partial mu antagonist analgesic, with some binding to the delta receptor and minimal agonist activity at the sigma receptor.

Adverse reaction **:**

- Cardiovascular: Hypertension, hypotension, bradycardia, tachycardia, pulmonary edema.
- Gastrointestinal: Cramps, dyspepsia, bitter taste.
- Respiration: Depression, dyspnea, asthma.
- Dermatological: Itching, burning, urticaria.
- Obstetric: Pseudo-sinusoidal fetal heart rhythm.

Dose **:** 10 – 20mg

Pharmacokinetics

Mean plasma nalbuphine concentrations 5 min after intravenous administration of 10 or 20 mg were 39 and 73 ng/ml, respectively. The mean maximum plasma concentrations (Cmax) after intramuscular or subcutaneous administration of nalbuphine 10 mg were 29 and 31 ng/ml, respectively. Mean Cmax values after 20 mg doses were 60 and 56 ng/ml. Mean Cmax occurred 30 to 40 min after nalbuphine administration. The mean elimination half-lives of parenterally administered nalbuphine ranged between 2.2 and 2.6 h, regardless of dose given or route administered. The mean absolute bioavailability was 81% and 83% for the 10 and 20 mg intramuscular doses, respectively, and 79% and 76% following 10 and 20 mg of subcutaneous nalbuphine. The mean volumes of distribution (Vss) of the intravenously administered drug were 290 and 274 l and the mean systemic clearances were 1.6 and 1.5 l/min following administration of 10 and 20 mg doses, respectively. Intramuscular and subcutaneous nalbuphine appear to be interchangeable based on the similarities in Cmax, mean times until maximum concentration, mean AUC data, and absolute bioavailabilities.

Pharmacodynamics

Nalbuphine is a synthetic opioid agonist-antagonist analgesic of the phenanthrene series. Nalbuphine's analgesic potency is essentially equivalent to that of morphine on a milligram basis. The opioid antagonist activity of nalbuphine is one-fourth as potent as nalorphine and 10 times that of pentazocine. Nalbuphine by itself has potent opioid antagonist activity at doses equal to or lower than its analgesic dose. When administered following or concurrent with mu agonist opioid analgesics (e.g.,

morphine, oxymorphone, fentanyl), nalbuphine may partially reverse or block opioid-induced respiratory depression from the mu agonist analgesic. Nalbuphine may precipitate withdrawal in patients dependent on opioid drugs. Nalbuphine should be used with caution in patients who have been receiving mu opioid analgesics on a regular basis.

MATERIALS AND INSTRUMENTS

Instruments used:

- System : HPLC Prominence Waters-2695
- Pump : I80 (LC – 10 AT Vp series)
- Detector : UV/Visible E2469
- Column : Waters C_8 column. (150mm x 4.6 mm, 5 µ i.d.)
- pH meter : Elico
- vacuum pump : Gelman science
- Digital balance : Sartorious BSA224S-CW
- Sonicator : PCI Analytics

Reagents and Chemicals

Sodium Octane Sulphonate **:** AR grade

Sodium Acetate Trihydrate **:** AR grade

Glacial acetic acid **:** AR grade

Methanol **:** AR grade

Water **:** HPLC grade

Reference Standards

Nalbuphine hydrichloride **:** % purity 94.1

Preparation of solvents

Preparation of buffer:

Preparation of Buffer

Dissolve 1.08 g sodium octane sulphonate and 23.8 g of sodium acetate trihydrate in 550ml of water.

Add 1.0ml triethylamine and 450 ml of methanol, and mix. Adjust with glacial acetic acid to a pH of 6.5±0.1. Filter and degas prior to use.

Injection Brand Used:

ASMITHA TABLETS

Label claim:

1.Nalbuphine HCl : 10 mg/mL

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

SOLUBILITY

According to literature, Nalbuphine HCl soluble in soluble in Water (35.5 mg/mL at 25ºC and Ethanol, insoluble in Chloroform and ether.

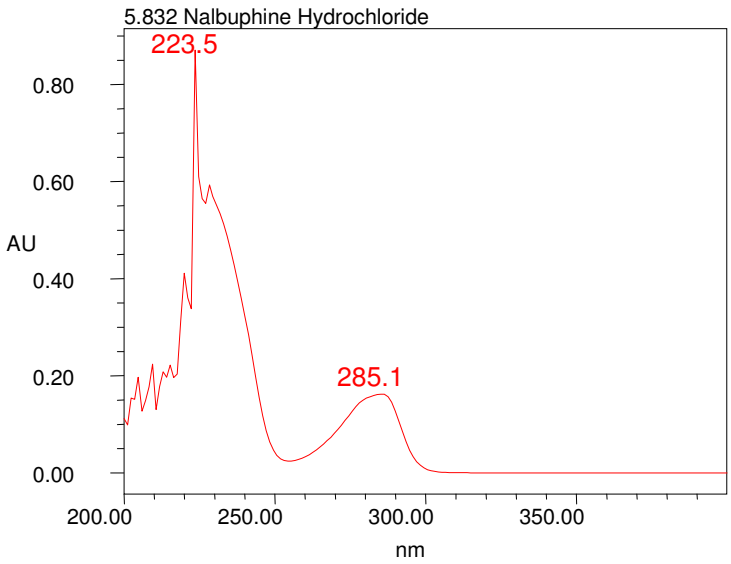
SELECTION OF CHROMATOGRAPHIC CONDITION

Proper selection of the method depends upon the nature of the sample (ionic / ionisable / neutral molecule), its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

SELECTION OF WAVELENGTH (λ max)

In setting up the conditions for the development of the assay method, the choice of detection wavelength was based on the scanned absorption for *Nalbuphine HCl*. The spectrum was scanned over the range of 190 – 400 nm and was obtained by measuring the absorption of 1.0 mg/ml solution of Nalbuphine HCl in Water, prepared from stock solution. The spectrum was obtained by using 1cm quartz cell using water as reference solution. λ_{max} of *misoprstol* was 280. Hence for estimation 280 nm was selected.

Figure 6: UV – spectrum of Nalbuphine HCl



7.1. METHOD DEVELOPMENT TRIALS

Trial –1

Prapration of solutions:

Mixed 0.05% trifluoroacetic acid, acetonitrile, and tetrahydrofuran

Preparataion of mobile phase:

Mixed 350ml of 0.05% trifluoroacetic acid and 550 ml of acetonitrile and 100 ml of tetrahydrofuran shekwell and filtered through 0.45µ membrane filter degassed prior to use

Mobile phase Composition : Isocratic 1.0ml/min

Stationary Phase : Thermo Hypersil BDS C_{18}, 250mm x 4.0mm, 5µm

Mobile phase ratio :35:55:10Trifluoroacetic acid:Methanol:Tetrahydrofuran

Flow rate : 1.0 ml

Column temperature : 25^0C

Selected wave length : 280 nm.

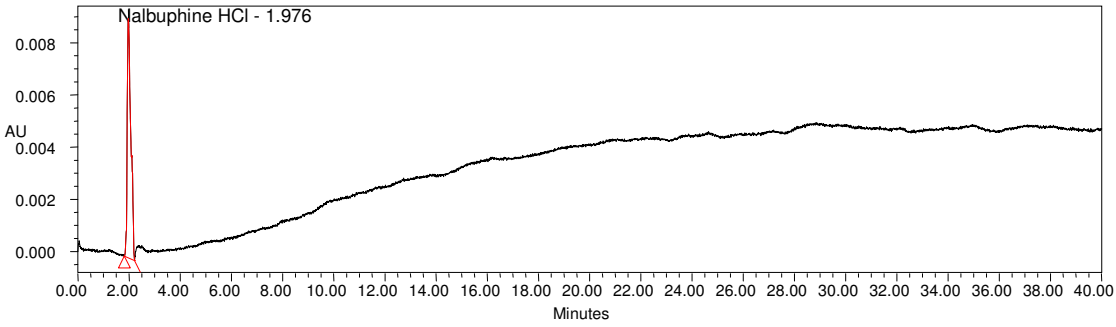
Injection Volume : 20 µl

Run Time : 20 min.

Result:

While injecting the above chromatographic condition, nalbuphien HCl RT was found identified separately.but it . as found to be more noise in the base line and pH of the mobile phase also need to change. and RT of the compound is 1.976.

Chromatogram 1



Trial –2

Preparation of Mobile Phase

Dissolve 1.08 g sodium octane sulphonate and 23.8 g of sodium acetate trihydrate in 550ml of water.

Add 450 ml of methanol, and mix. Adjust with glacial acetic acid to a pH of 6.5±0.1. Filter and degas prior to use.

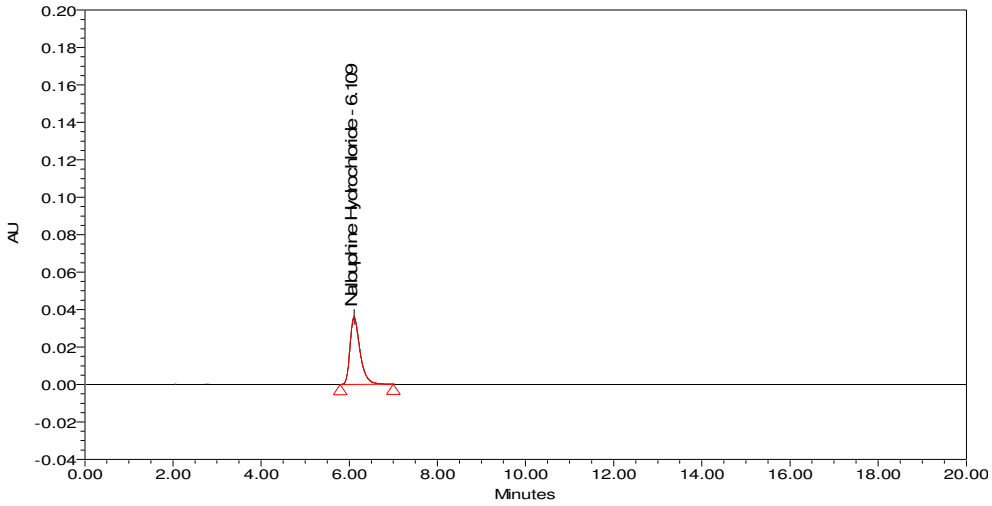
Mobile phase Composition : Isocratic 1.0ml/min

Stationary Phase 5µm Mobile phase ratio	:	Thermo Hypersil BDS C_{18}, 250mm x 4.0mm, : 55:45 Buffer:Methanol
Flow rate	:	1.0 ml
Column temperature	:	25^0C
Selected wave length	:	280 nm.
Injection Volume	:	20 µl
Run Time	:	20 min.

Result:

While injecting the above chromatographic condition, nalbuphien HCl RT was found identified separately. But the tailing of the compound is 2.1. but as per the guidelines tailing more than 2.0 is not acceted. So tailing need to be minimize.

Chromatogram 2



TRIAL - 3

Preparation of Mobile Phase

Dissolve 1.08 g sodium octane sulphonate and 47.2 g of sodium acetate trihydrate in 550ml of water.

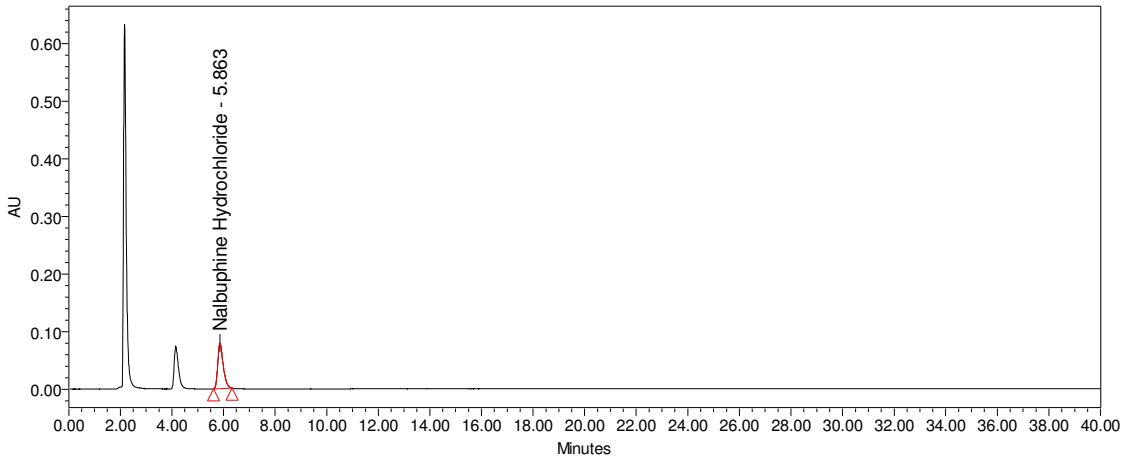
Add 450 ml of methanol, and mix. Adjust with glacial acetic acid to a pH of 6.5±0.1. Filter and degas prior to use.

Mobile phase Composition : Isocratic 1.0ml/min

Stationary Phase	:	Thermo Hypersil BDS C_{18}, 250mm x 4.0mm, 5µm Mobile phase ratio : 55:45 Buffer:Methanol
Flow rate	:	1.0 ml
Column temperature	:	25^0C
Selected wave length	:	280 nm.
Injection Volume	:	20 µl
Run Time	:	20 min.

Result:

While injecting the above chromatographic condition, nalbuphien HCl RT was found identified separately. But the tailing of the compound is 1.2. the response due to blank and placebo peak is high compare to the main peak. This should be minimize by changing the mobile phase.



TRIAL –4

Stationary Phase : Thermo Hypersil BDS C_{18}, 250mm x 4.0mm, 5µm Mobile phase ratio : 55:45 Buffer:Methanol

Flow rate : 1.0 ml

Column temperature : 25^0C

Selected wave length : 280 nm.

Injection Volume : 20 µl

Run Time : 20 min.

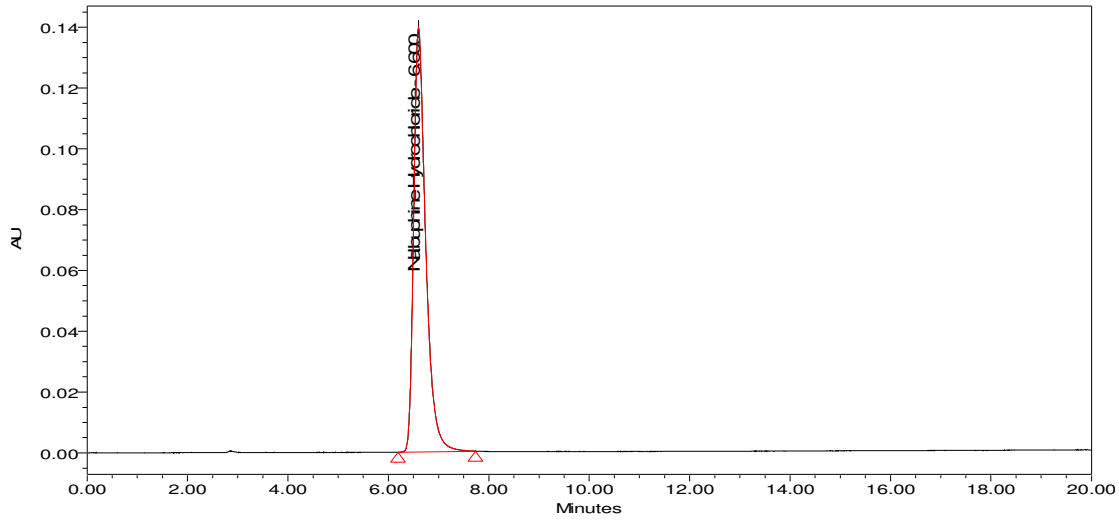
PREPARATION OF SOLUTIONS

Preparation of Mobile Phase

Dissolve 1.08 g sodium octane sulphonate and 23.8 g of sodium acetate trihydrate in 550ml of water.

Add 1.0ml triethylamine and 450 ml of methanol, and mix. Adjust with glacial acetic acid to a pH of 6.5±0.1. Filter and degas prior to use.

Chromatograms 4



OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Stationary Phase : Thermo Hypersil BDS C_{18}, 250mm x 4.0mm, 5µm Mobile phase ratio : 55:45 Buffer:Methanol

Flow rate : 1.0 ml

Column temperature : 25^0C

Selected wave length : 280 nm.

Injection Volume : 20 µl

Run Time : 20 min.

PREPARATION OF SOLUTIONS

Preparation of Mobile Phase

Dissolve 1.08 g sodium octane sulphonate and 23.8 g of sodium acetate trihydrate in 550ml of water.

Add 1.0ml triethylamine and 450 ml of methanol, and mix. Adjust with glacial acetic acid to a pH of 6.5±0.1. Filter and degas prior to use.

Diluent

Mobile phase used as diluent

Standard preparation

Weigh accurately about 20 mg of Nalbuphine Hydrochloride working standard and transfer into a 100 ml volumetric flask, then add 70 ml of diluent, sonicate for 5 minutes to dissolve and make up the volume with diluent.

Sample preparation

Select random of 20 Ampoules. Transfer the content into the 50 ml beaker. Pipette out 5 ml of sample solution (equivalent to about 50 mg of Nalbuphine Hydrochloride), and transfer into a 250mL volumetric flask, add 170 mL of diluent, and make up the volume with diluent.

Pipette out 5 ml of solution, and transfer into a 250mL volumetric flask, add 170 mL of diluent, and make up the volume with diluent.

VALIDATION OF RP-HPLC METHOD

After development of HPLC method for the estimation of the Single component dosage forms validation of the method was carried out. This section describes the procedure followed for the validation of the developed method.

SYSTEM SUITABILITY STUDIES

System suitability studies were carried out as specified in the United States Pharmacopoeia (USP). These parameters include column efficiency, resolution, tailing factor and RSD were calculated in present study.

System Suitability Parameters	Observed value	Acceptance criteria
The Tailing factor for Nalbuphine Hydrochloride peak from first injection of standard preparation.	**1.5**	**NMT 2.0**
Theoretical plate count for Nalbuphine Hydrochloride from first injection of standard preparation.	**3853**	**NLT 1500**
The % RSD of six replicate injections for Nalbuphine Hydrochloride of standard preparation.	**0.1**	**NMT 2.0**

Table No: 1 System suitability parameters

Specificity

The following methods were employed for demonstrating specificity for HPLC method. In the first method, the conditions of HPLC method developed, namely, percentage of the organic solvent in mobile phase, pH of the mobile phase, flow rate, etc. were changed in HPLC and the presence of additional peaks, if any, was observed. The second method involves the peak purity test method using diode array detector. The diode array derivative spectrums and derivative chromatograms of the standard and sample drug peaks were recorded and compared. The third method was based on measurement of the absorbance ratio of the drug peaks at different wavelengths.

Table No 2: Specificity

Sample ID	Interference		
	Nalbuphine Hydrochloride RT	**Purity Angle**	**Threshold angle**
Blank	**NIL**	**NAP**	**NAP**
Placebo	**NIL**	**NAP**	**NAP**
Standard+Placebo	**6.123**	**0.15**	**0.304**
Standard	**6.122**	**0.109**	**0.291**
Test Sample	**6.123**	**0.15**	**0.304**

Accuracy

Accuracy of the method was determined by recovery experiments. To the formulation, the reference standards of the respective drugs were added at the level of 100 %. These were further diluted by procedure as followed in estimation of formulation. The resulting sample solutions were analyzed by HPLC. The amount of the each drug present, percentage recovery, percentage relative standard deviation (% RSD) was calculated. The percentage recovery was calculated using the formula,

$$\text{Percentage recovery} = \frac{[a+b]-a}{b} x\,100$$

Table No: 3 Recovery Studies

Series	No of Sample	Added in mg	Found in mg	Recovery in %	Average in %
50%	**01**	26.65	26.35	98.9	**99.3**
	02	26.58	26.41	99.4	
	03	26.42	26.35	99.7	

75%	**01**	39.52	39.79	100.7	**100.5**
	02	39.57	39.89	100.8	
	03	39.66	39.70	100.1	
100%	**01**	51.32	52.18	101.7	**101.2**
	02	51.63	52.15	101.0	
	03	51.86	52.32	100.9	
125%	**01**	65.16	65.51	100.5	**100.8**
	02	64.84	65.54	101.1	
	03	65.08	65.65	100.9	
150%	**01**	78.16	78.56	100.5	**100.7**
	02	77.80	78.54	100.9	
	03	77.79	78.35	100.7	
			Mean	**100.5**	
			Stdev.	**0.72**	
			% RSD	**0.7**	
			Confidence Interval	**100.2 & 100.9**	

Precision

Table No: 5 System Precision of Nalbuphine HCl

No of injection	RT	Response
01	6.600	2328333
02	6.594	2329431
03	6.596	2325802

04	6.594	2326325
05	6.594	2323823
06	6.592	2326956
Mean	**6.595**	**2326778**
SD	**0.00**	**1968.65**
% RSD	**0.0**	**0.1**

System Suitability Parameters	**Observed value**	**Acceptance criteria**
The Tailing factor for Nalbuphine Hydrochloride peak from first injection of standard preparation.	**1.5**	**NMT 2.0**
Theoretical plate count for Nalbuphine Hydrochloride from first injection of standard preparation.	**3853**	**NLT 1500**
The % RSD of six replicate injections for Nalbuphine Hydrochloride of standard preparation.	**0.1**	**NMT 2.0**
The % RSD of Nalbuphine Hydrochloride peak RT for six injection of standard solution.	**0.0**	**NMT 1.0**

Test Preparation

No. of Sample	**Method Precision**
	Nalbuphine Hydrochloride
01	99.3
02	99.3
03	99.5

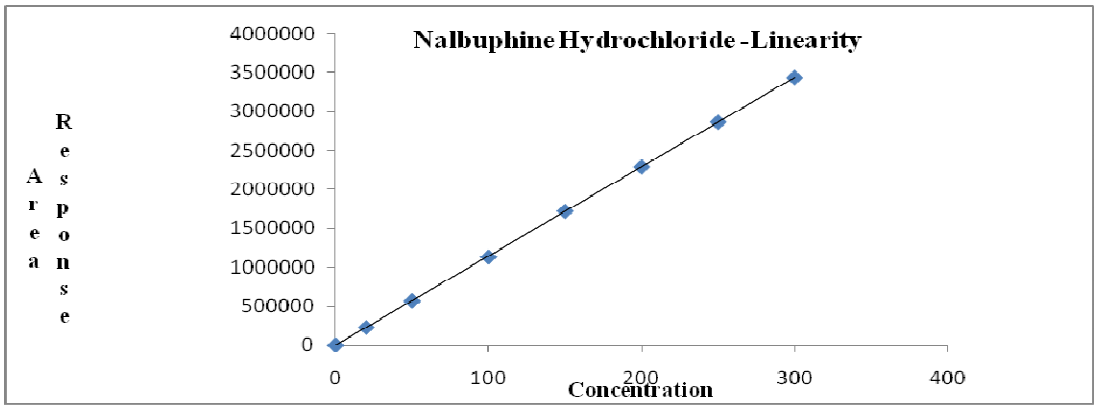
04	99.7
05	99.4
06	99.5
Mean	**99.4**
SD	**0.16**
% RSD	**0.2**
Confidence Interval	**99.3 & 99.6**

Linearity and range

Linearity was established by plotting a graph between concentration on X-axis and peak area on Y-axis and the correlation coefficient was determined. Seven different concentrations of Nalbuphine HCl concentration ranging from LOQ, 50%, 75%, 100%, 125%, 150% and 200% with respect to working concentration were prepared and analyzed as per test method. The results are summarized in the table given below.

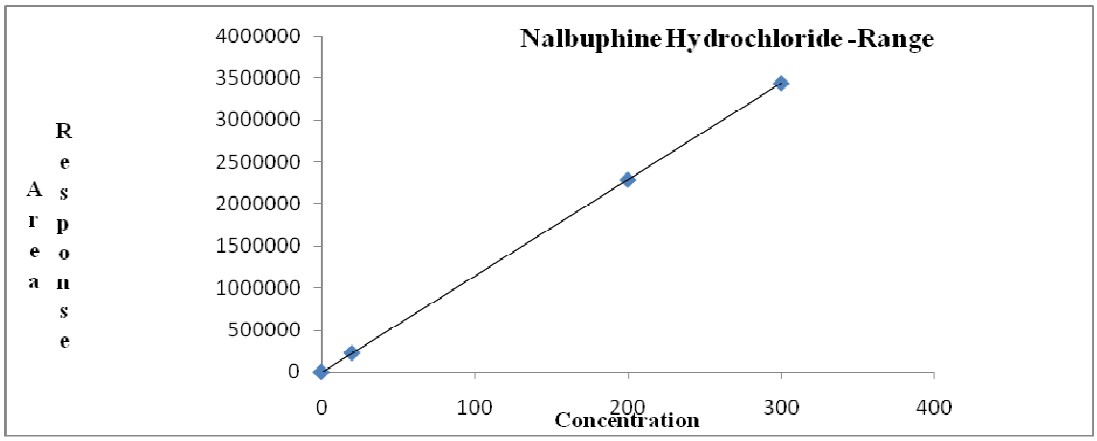
S. No.	% Level	Concentration in µg/ml (ppm)	Peak Response of Nalbuphine Hydrochloride
01	**10%**	20.00	226809
02	**25%**	50.00	568281
03	**50%**	100.00	1133345
04	**75%**	150.00	1719578
05	**100%**	200.00	2290890
06	**125%**	250.00	2868422
07	**150%**	300.00	3440805
		Slope	**11494**

Y intercept	**-7149**
Coefficient of correlation	**1.0000**
Coefficient of regression (r^2)	**1.0000**
Y intercept should be ±2.0% of the active response at 100% concentration	**-0.3**



Range

Level	Concentration in µg/ml	%RSD
Lower Level 10%	**20.00**	**0.2**
Middle Level 100%	**200.00**	**0.1**
Higher Level 150%	**300.00**	**0.1**



Ruggedness

Defined by the USP as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst.

Robustness

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

1. Effect of variation in Wavenlength variation
2. Effect of variation in Column Oven Temperature

The separation factor, retention times and peak symmetry were then calculated.

1. Table No: 11 Effect of Wavelength variation

System Suitability Parameters	Observed value at Wavelength			Acceptance criteria
	278 nm	280 nm	282 nm	
The Tailing factor for Nalbuphine Hydrochloride peak from first injection of standard preparation.	**1.5**	**1.5**	**1.5**	**NMT 2.0**
Theoretical plate count for Nalbuphine Hydrochloride from first injection of standard preparation.	**3677**	**3853**	**3603**	**NLT 1500**
The % RSD of six replicate injections for Nalbuphine Hydrochloride of standard preparation.	**0.3**	**0.1**	**0.2**	**NMT 2.0**

2. For Comparison between System -1 & System-2

No. of Sample	System -1	System-2
	Nalbuphine Hydrochloride	**Nalbuphine Hydrochloride**
01	99.3	99.6
02	99.3	99.3
03	99.5	99.1
04	99.7	99.2
05	99.4	99.1
06	99.5	99.2
Mean	**99.4**	**99.2**
SD	**0.16**	**0.19**
% RSD	**0.2**	**0.2**
Confidence Interval	**99.3 & 99.6**	**99.1 & 99.4**

18.2 Effect of variation in Column Oven Temperature

To demonstrate the robustness of test method, check the system suitability parameters by injecting standard preparations into the HPLC system at 35°C and at 45°C of Column oven temperature. Evaluate the system suitability parameters and tabulate the results in the table given below.

Table no: 12

System Suitability Parameters	Observed value with Column Oven Temperature			Acceptance criteria
	20°C	**25°C**	**30°C**	

The Tailing factor for Nalbuphine Hydrochloride peak from first injection of standard preparation.	**1.6**	**1.5**	**1.6**	**NMT 2.0**
Theoretical plate count for Nalbuphine Hydrochloride from first injection of standard preparation.	**3079**	**3853**	**3509**	**NLT 1500**
The % RSD of six replicate injections for Nalbuphine Hydrochloride of standard preparation.	**0.1**	**0.1**	**0.1**	**NMT 2.0**

Stability studies:

Stability of the sample and standard used in HPLC method is required for a reasonable time to reproducible and reliable results. The stability of the sample spiked with the drug was subjected to short term stability at room temperature after 8 hours.

Table No: 15 Refrigerator Solution Stability

Time in hours	Standard area	Sample area
Initial	2399731	2378739
4th Hours	2409544	2378739
8th Hours	2401025	2374541
12th Hours	2405911	2381950
16th Hours	2412544	2388625
20th Hours	2414694	2385761
24th Hours	2415554	2380472
28th Hours	2423670	2392169
32nd Hours	2426932	2393142
36th Hours	2412867	2388382
40th Hours	2422432	2390247

44th Hours	2417133	2394083
48th Hours	2406559	2384841
Mean	**2412969**	**2385515**
SD	**8400.2**	**6238.7**
% RSD	**0.3**	**0.3**

17.4 Filter Validation

Carry out filter validation using three different filters namely, 0.45 µm nylon, 0.45 µm PVDF and whatman No.1 compare with unfilter sample. Prepare the standard solution as per the test methods and similarly prepare the test preparation.

Filter the test preparation through individual filters. Inject standard solution and filtered test solution into the HPLC system under the chromatographic condition. Calculate the % difference between the assay obtained in different filters.

Filter description	**Filters**		
	0.45µm Nylon	**0.45 µm PVDF**	**Whatman No.1**
Manufacturer Name	Nupore Filtration Systems Pvt. Ltd.	Millipore	GE Healthcare Ltd
Lot No.	QNN 0939314	R3PA17924	1001-150
Size	0.45µm	0.45µm	1

Filters	**Assay in %**	**Difference from Initial**
Unfilter	**99.7**	**NAP**
0.45 µm nylon	**98.2**	**1.5**
0.45 µm PVDF	**99.7**	**0.0**
Whatman No.1	**100.4**	**0.7**

ASSAY OF PROPOSED METHOD:

Procedure:

Separately inject both the standard and sample preparations into liquid chromatogram and record the peak area responses. The % RSD is not more than 2.0.

a) Correlation Coefficient (r):

$$\frac{n \sum xy - \sum x \sum y}{\sqrt{\left(n \sum x^2 - [\sum x]^2\right)\left(n \sum y^2 - (\sum y)^2\right)}}$$

Σ = Sum of, x = Conc. of the Component, y= Average area of component,

n = number of observations

b) Slope (a) = $\frac{(n\sum XY - \sum X \sum Y)}{(n\sum X^2 - (\sum X)^2)}$

c) The equation of straight line: Y = a X + b

d) Intercept on the Y axis (b) = $\overline{Y} - a\overline{X}$ **,** ($\overline{X}$ = mean values of *X*,)

($\overline{Y}$ = Mean values of Y)

e) Calculation

Calculate the mg of Nalbuphine Hydrochloride in the injection:

Ru X Ws X 250 X P X 1

Rs X 100 X V X 100

=Content in mg/ml

Calculate the Nalbuphine Hydrochloride content in percentage of the label claim:

$$\text{Content in mg} = \frac{\text{Content in mg}}{\text{Label claim}} \times 100$$

Where,

Ru= Peak response for Nalbuphine Hydrochloride from the sample solution

Rs= Peak response for Nalbuphine Hydrochloride from the standard solution

Ws=Standard weight

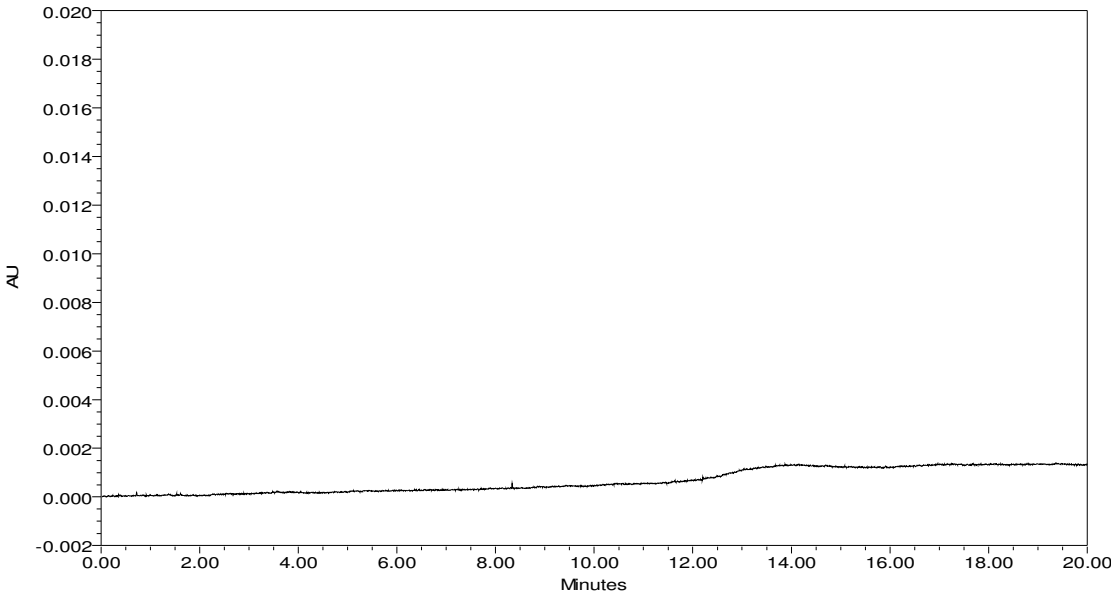
V= 5 ml of the sample

P=Purity in as such basis.

8. CHROMATOGRAMS

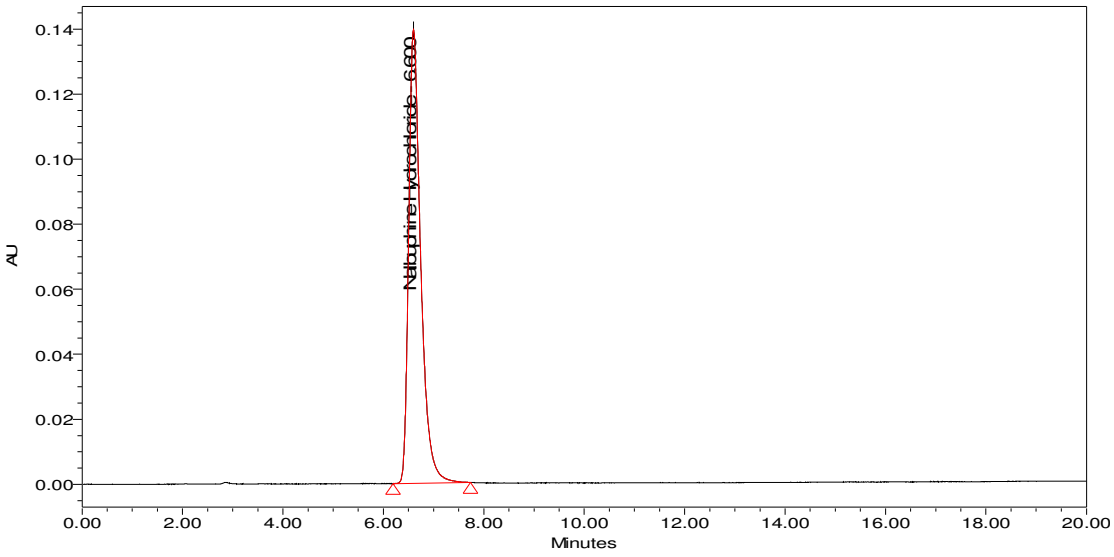
Chromatogram No`:1

A Representative chromatogram of BLANK



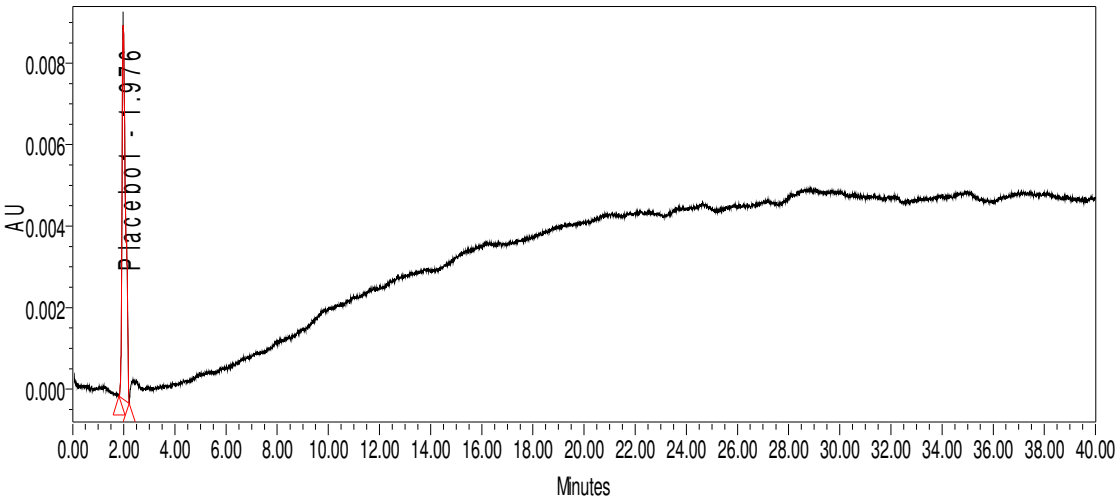
Chromatogram No: 2

A Representative chromatogram of STANDARD NALBUPHINE HCL



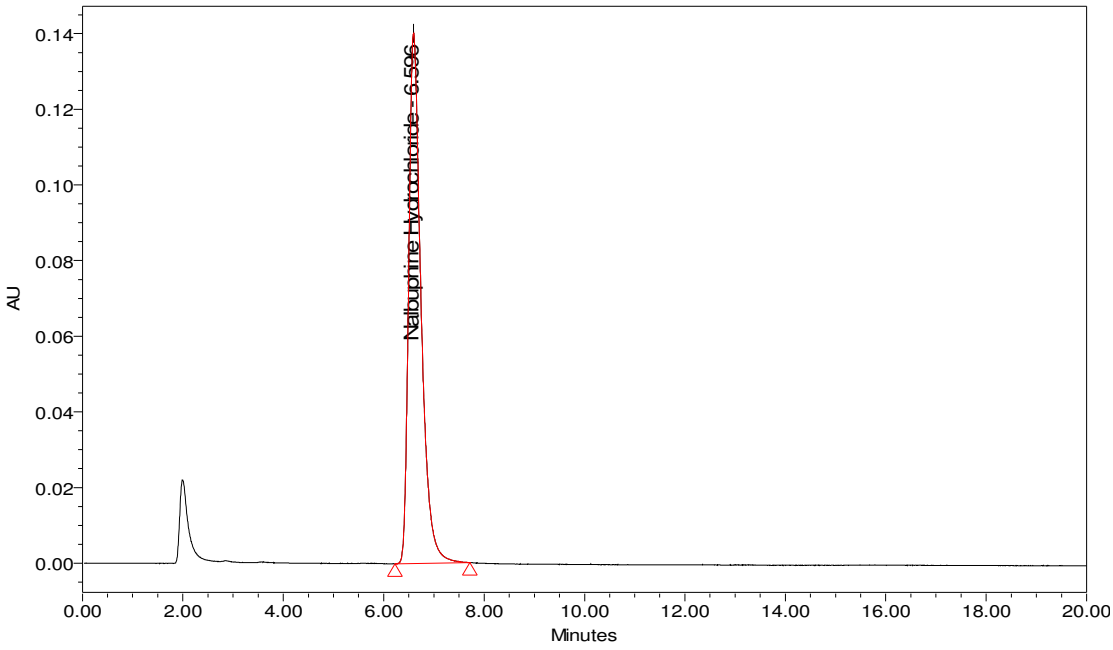
Chromatogram No: 3

PLACEBO NALBUPHINE HCL

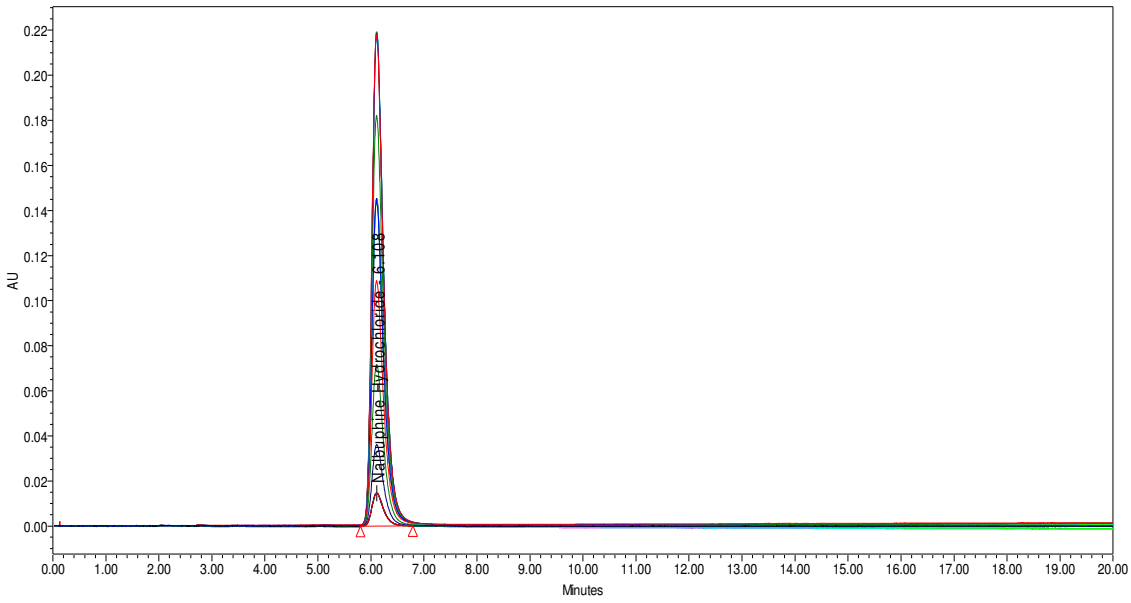


Chromatogram No: 4

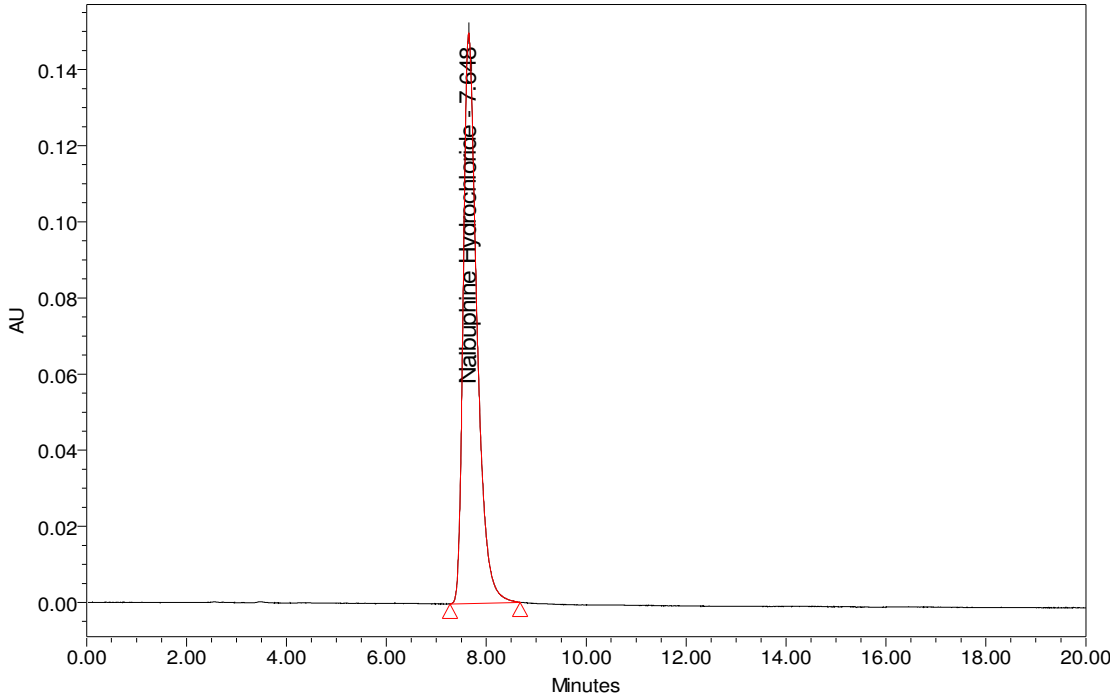
A Representative chromatogram of SAMPLE NALBUPHINE HCL



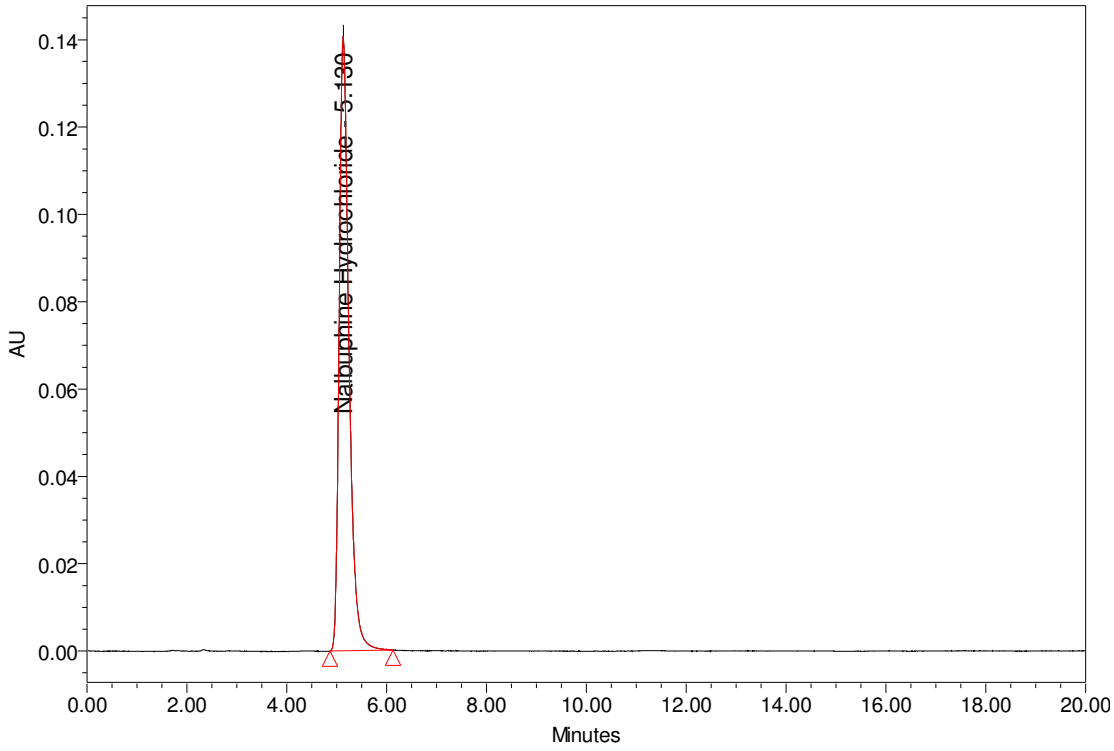
A Representative chromatogram of the linearity 10%-150% solution



Low Flow



High flow


Nalbuphine Hydrochloride - 5.130
AU
Minutes
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

RESULT AND DISCUSSION

VALIDATION OF THE METHOD

The suitability of the system was studied by the values obtained for Theoretical plate, Resolution and tailing factor, %RSD of the chromatogram of standard drugs and presented in the table(1). The selectivity of the method was revealed by the repeated injection of mobile phase and no interference was found and presented in Table (2)

The accuracy of the method was determined by recovery experiments. The recovery studies were carried out by preparing 3 individual samples with same procedure from the formulation and injecting. The percentage recovery and percentage relative standard deviation of the percentage recovery was calculated and presented in Tables (3) & (4). From the data obtained, added of standard drugs were found to be accurate.

The precision of the method was demonstrated by system and method precision. All solutions were injected into the chromatographic system. The peak area and percentage relative standard deviation were calculated and presented in tables (5) & (6).

The linearity of proposed method were performed by using the concentration range of 10 % to 150 % of standard concentration i.e 20 µg/ml to 200 µg/ml of *Nalbuphine HCl* was presented in Table 7, 8 , 9& 10 .The response factor, slope, intercept and correlation co-efficient were calculated. The slope, intercept, correlation co-efficient were found to be 11497, 7149, 1.0000. The calibration curves were plotted using response factor (Vs) concentration of standard solutions (fig: 6 &7). The calibration graph shows that linear response was obtained over the range of concentration used in the procedure. These data demonstrates that the method have adequate sensitivity to the analytes. The range demonstrate that the method is linear outside the limits of expected use.

The robustness of the method was studied by carrying out experiments by changing conditions discussed earlier. The response factors for these changed chromatographic parameters were almost same as that of the fixed chromatographic parameters (table11&12) and hence developed method is said to be robust and ruggedness performed by analyst 1 and analyst 2 .

The stability studies were carried out at zero hour and after 48 hour, results were tabulated in table 15.

VALIDATION PROTOCOL SUMMARY

The following is the summary of Analytical method validation study conducted for Assay of Nalbuphine Hydrochloride in Nalbuphine injection 10 mg/mL.

- System Suitability and System Precision is established as mentioned in the test method.
- The test method is specific for the estimation of Nalbuphine Hydrochloride in Nalbuphine injection 10 mg/mL.
- There is no interference or co-elution of degradants in quantifying the Nalbuphine Hydrochloride in Nalbuphine injection 10 mg/mL.
- There test method is meeting Method Precision and Intermediate Precision acceptance criteria.
- The method performance at lower to higher levels (10 % to 150%) is Linear, Precise and Accurate.
- The detector response is linear with a coefficient of correlation 1.0000 and coefficient of regression (r^2) 1.0000 for Nalbuphine Hydrochloride.
- Range is found within the limit.
- The method is found Linear, Precise and Accurate.
- The test method is rugged for system to system, analyst to analyst.
- The standard solutions and test solution was stable at bench top for a period of 48 hours.
- The allowable variation in column temperature is from 20°C to 30°C.
- The allowable variation in Flow Rate is from 0.8 ml/min to 1.2 ml/min.
- The allowable variation in Wavelength Change is from 278 nm to 282 nm.

- The Unfilter, 0.45 µ Nylon and 0.45 µ PVDF, Whatman No.1 are suitable for performing filtration.

SUMMARY AND CONCLUSION

From the reported literature, there were few methods established for the determination of Nalbuphine HCl.

It was concluded that there was few method reported for the estimation of the above selected component dosage form, which promote to pursue the present work. The scope and object of the present work is to develop and validate a new simple HPLC method for estimation of Nalbuphine HCl in injection dosage form.

In RP-HPLC method development, the estimation was carried out by using the Thermo Hypertsil C_{18} column (4.6 X 250 mm) with 5-micron particle size. Injection volume of 50µl is injected and eluted with the mobile phase Sodium Acetate Buffer, Methanol with the ratio of 55: 45, which is pumped at the flow rate of 1.0 ml / min. Detection was carried out at 280 nm. Quantitation was done by calibration curve method with the above mentioned optimized chromatographic condition. This system produced symmetric peak shape, good resolution and reasonable retention times of Nalbuphine HCl were found to be 6.109 minutes.

The 20 µg/ml to 200 µg/ml of *Nalbuphine HCl*. The slope intercept and correlation coefficient(s) were found to be, 11497, 7149, 1.0000 for *Nalbuphine HCl* which indicates excellent correlation factor Vs concentration of standard solutions.

Precision of the developed methods was studied under system precision, method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The %RSD values for recovery percentage of *Nalbuphine HCl* was found to be within the acceptable criteria. The result indicates satisfactory accuracy of method for estimation of the above mentioned drugs.

Hence, the chromatographic method developed for *Nalbuphine HCl* are rapid, simple, specific, sensitive, precise, Accurate. The RP-HPLC was simple and

does not suffer from common excipients in pharmaceutical preparation and highly useful in the analysis of drugs in pharmaceutical formulation.

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